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DARPins and other repeat protein scaffolds: advances in engineering and applications

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Antibodies have long been regarded as the only class of binding proteins. With the emergence of protein engineering techniques, new binding proteins based on alternative scaffolds have been designed. Additionally, modern technologies for selection and evolution from libraries are independent of the antibody scaffold and could thus be readily used for obtaining specific binding proteins. One important group of alternative scaffolds is based on repeat proteins. Nature is widely using these proteins to modulate protein–protein interactions, and even in the adaptive immune system of jawless vertebrates; the step to their application as an alternative to antibodies seems therefore logical. In this review, progress on DARPins and other repeat protein scaffolds will be discussed. Advances in their design as well as novel applications will be highlighted.

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Introduction

Traditionally, monoclonal antibodies have been used for most applications where a specific protein binding with high affinity to its target was needed. Yet, the advent of synthetic libraries and selection and evolution technologies has not only made immunization unnecessary, but has also made the antibody molecule itself dispensable.

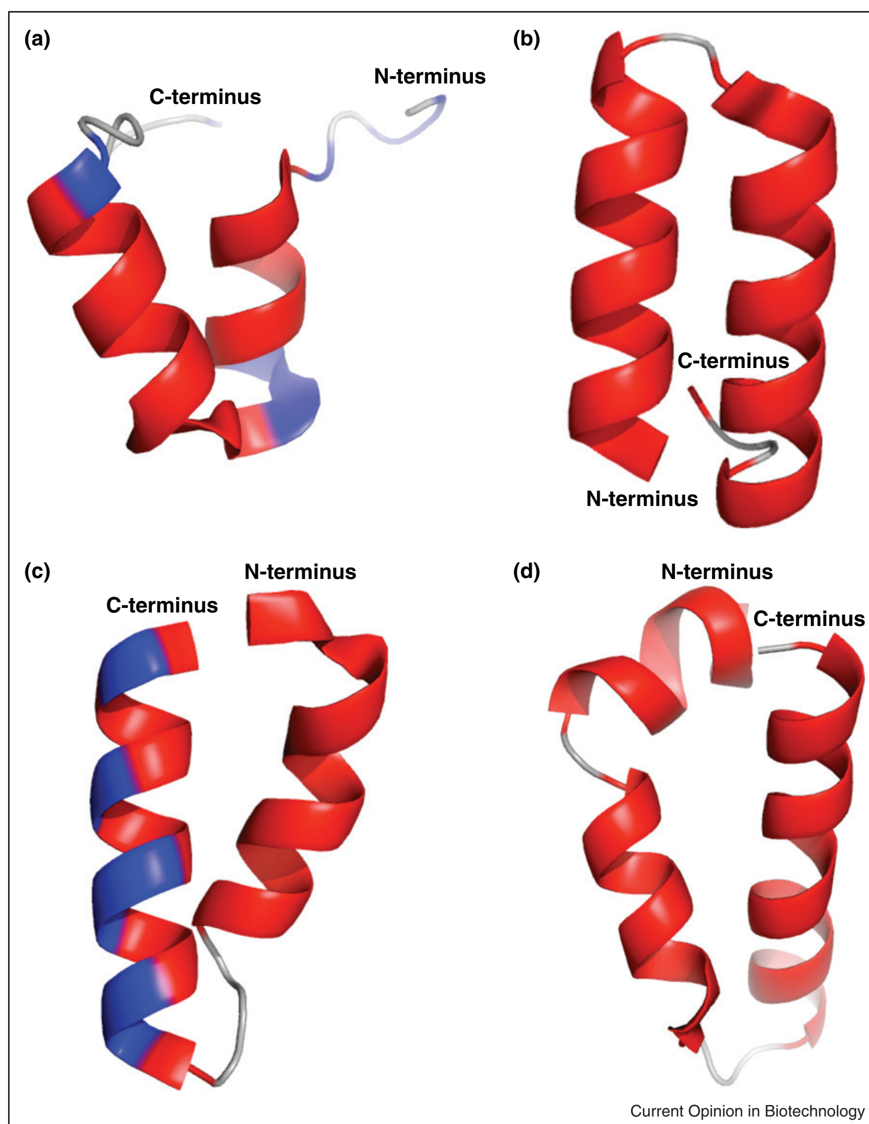
New scaffold proteins that should take on the role of antibodies and expand their range of applications ideally should surpass antibodies in biophysical properties. A wish list can be formulated: first, the absence of aggregation not only favors an efficient selection process but is also required for practically all *in vitro* applications — and absolutely mission-critical for *in vivo* applications. Second, many applications require chemical coupling, for example, to fluorophores, toxins, nanoparticles or solid supports. This is much facilitated if the protein can be engineered to have a unique cysteine. Third, the absence of disulfides is a prerequisite for functional expression in the cytoplasm of *Escherichia coli*; correct folding in the cytoplasm of higher cells will also allow functional studies. Fourth, multivalence and multispecificity are a very generic concepts to increase avidity, or to crosslink different epitopes or targets. Multimeric assemblies should ideally have the same expression yield as a monomer. Furthermore, different linking geometries should be realized with ease. Fifth, scaffolds should be able to give rise to well expressing fusion proteins that do not lead to additional aggregation. Sixth, scaffolds should allow convenient engineering for *in vivo* half-life extension; for example, by site-specific PEGylation, or by fusion to a domain that binds to serum proteins with long half-lives themselves.

Over the last few years, a number of novel alternative binding molecules have been designed [1–4], which address these issues with varying degrees of success. In this review, scaffolds based on naturally occurring repeat proteins will be discussed as a promising platform for novel applications.

Nature has applied repeat proteins as scaffolds to support a large repertoire of different protein–protein interactions [5–10]. Most remarkable, in jawless vertebrates, the adaptive immune system is not based on the immunoglobulin fold, but on the leucine-rich repeat (LRR) family [11], which are diversified and naturally selected from a repertoire. Repeat proteins are thus considered by nature as ‘antibodies’.

Repeat proteins are characterized by small, repeating structural motifs of 20–50 amino acids; the number varies between families. The motifs or repeats stack together to form elongated stable structures, which allows for a larger surface area — and thus a larger potential binding area — than typical globular proteins [12,13]. The elongated structure is stabilized by hydrophobic interactions and hydrogen bonds, usually both within a repeat and between adjacent repeats. Many structures also have special repeats at the N-terminus and C-terminus of the protein with a more hydrophilic surface: these function as a ‘cap’ to shield the hydrophobic core from the solvent. This modular structure introduces numerous possibilities for binding a diverse set of ligands in a specific manner. The extended rigid surface suggests that the repeat protein

Figure 1



Cartoon representations of the repeats discussed in this review. **(a)** A DARPin internal repeat (PDB code 2XEE, [25]). Randomized residues are depicted in blue. **(b)** Representation of a consensus designed TPR repeat (PDB code 1NAO, [31]). **(c)** The αRep-4 repeat based on the HEAT-like proteins (PDB code 3LTJ, [17••]). Randomized residues are depicted in blue. **(d)** The ARM repeat structure, based on the model of the Y3MA mutant [18].

loses little entropy upon binding the target: provided a library is large enough and well designed, very high affinities can be obtained.

In this review, recent advances in the design and engineering of a number of repeat proteins will be discussed (Figure 1 and Table 1). Specific examples of novel applications of designed repeat proteins will be highlighted.

Consensus design

The successful design of full consensus LRRs [14], ankyrin (AR) [15], and tetratricopeptide (TPR) repeats has been described and discussed in previous reviews [13,16]. More recently, the design of consensus armadillo (ARM) and HEAT repeat proteins has been reported [17••,18].

Briefly, there are two motivations for using consensus design, as opposed to using one particular repeat protein family member as a scaffold and randomizing its surface: first, the consensus design, if done correctly, can markedly increase stability of the library members, since stabilizing structural features inherent to a natural family but lost in individual natural proteins will be preserved [19] and second, consensus design makes repeats compatible, allowing addition, deletion, and exchange of repeats.

Table 1

Characteristics of repeat proteins.

Repeat protein scaffold	Amino acids/repeat[a]	Structural motif
HEAT (αRep)[b]	31	Two α-helices
Armadillo[c]	42	Three α-helices
TPR[d]	34	Helix-turn-helix
Ankyrin (DARPin)[e]	33	Helix-turn-helix-β-hairpin

[a] As used in the respective library design. In natural repeat proteins, greater variations have been observed.
[b] The HEAT repeat domain derives its name from the fact that the first members have been found in four proteins: huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast PI3-kinase TOR1 [76].
[c] In Drosophila genetics genes can be named by the appearance of the mutant larvae. The segment polarity gene β-catenin was the first member of this family to be discovered, and when deleted gave rise to Drosophila larvae with an appearance of an armadillo [77].
[d] The tetratricopeptide (TPR) repeat domain derives its name from the 34 amino acids that form the repeat. It was first described in the yeast cell division control protein 23 (CDC23) [78].
[e] The ankyrin repeat domain was first discovered as a repeated sequence in yeast cell-cycle regulation [79]. It is named after ankyrin, a cytoskeletal adapter protein, which was later found to contain 24 tandem copies of the repeat [80].

Furthermore, the design of full-consensus proteins in which every repeat has the same sequence can help to unravel principal features of the protein architecture which is important for subsequent engineering.

Designed ankyrin repeat proteins

In the case of the AR fold, two different design approaches were undertaken [15,20,21]. Mosavi *et al.* did not include a special capping repeat in their design, which resulted in proteins expressed in inclusion bodies [20]. In contrast, Binz *et al.* did include N- and C-terminal caps in the initial design, which both originated from the guanine–adenine-binding protein [15,21]. Indeed, the capping repeats were subsequently shown to be essential for efficient folding in the cell and for avoiding aggregation [22].

While the first DARPins were directly generated in a library format [15], full-consensus DARPins with one to six identical internal repeats were subsequently designed to study fundamental properties [23]: randomized positions were fixed according to sequence statistics and structural considerations, leading to a somewhat different consensus sequence than in [20].

As with the CTPRs and ARM repeats (see below), stability increases with the number of repeats: Full-consensus DARPins with more than three internal repeats are even resistant to denaturation by boiling or guanidine hydrochloride [23]. The crystal structure of the full-consensus DARPin with three internal repeats suggests that salt bridges on the surface are contributing to this additional thermal stability of the full-consensus proteins [24].

Molecular dynamics (MDs) simulations were carried out on proteins with one to five internal repeats [22]. The C-cap (in the original version taken from the GA protein) was the first to denature in almost all high-temperature simulations and is thus considered a limiting factor in the protein's otherwise superior stability. From these simulations, cap mutations with five or eight mutations and an extension of the second helix were suggested to improve its stability, which was subsequently experimentally verified [22]. This redesign significantly improved the packing of the C-cap to the core structure, as was confirmed by the 3D structures of three C-cap mutants [25]. Hydrogen/deuterium (H/D)-exchange experiments, measured by NMR, were performed with DARPins with two and three identical internal repeats (NI_2C and NI_3C), and an NI_3C with the reengineered C-cap (Mut5). The NI_3C_Mut5 variant was kept for one year at 37 °C, when some protons still had not exchanged, highlighting the extraordinary stability of the proteins. Importantly, these data emphasized that the better packing of the C-cap against the core in NI_3C_Mut5 led to a slower H/D-exchange throughout the whole protein molecule [26•]. This indicates that the redesigned C-cap bestows higher stability on the protein by making it into a larger cooperative unit.

HEAT and ARM repeats

HEAT repeat proteins are a rather diverse family with highly varied sequences [7]. This rather complicates consensus design: it would increase the risk of incorporating features from different subfamilies which are not compatible within a single consensus. Urvoas *et al.* therefore focused on a specific subtype of HEAT repeat proteins for their consensus design [17••]. The structure of Mth187 from *Methanobacterium thermoautotrophicum*, a protein with currently unknown function [27], was taken as the starting point for the alignment. This structure has one clear C-cap domain, but since the N-cap is not folded, a new N-cap was designed. After library construction, unselected variants with varying repeat numbers showed that the proteins were stable, well expressed and generally monomeric. However, a variant with four internal repeats (termed αRep-*n*4-a) showed dimeric behavior in size exclusion chromatography. This was confirmed by the elucidation of the 3D structure. Two different conformations (one obtained without, and one with bound PEG used as a precipitant in crystallization) indicated some flexibility within this scaffold and at the interface between monomers. Taken together, these findings provide a promising starting point for the future selection of specific binders against molecular targets.

Distantly related to the HEAT repeat protein is the ARM repeat protein [28,29]. Parmeggiani *et al.* designed an ARM-based scaffold for the generation of peptide-specific binders in a conserved orientation [18], with the vision of eventually creating a modular binding mode for peptides. The attractive feature of natural ARM repeat proteins is that peptides are bound in an antiparallel fashion to the

succession of repeats. A consensus design approach was initially followed, which led to stable and well-expressed proteins; however, the very first version had either formed dimers or molten globules. The molten globule variant was further optimized by a computational approach to stabilize the hydrophobic core, leading to native-like behavior in biophysical measurements [18]. Meanwhile, the framework has undergone several more design cycles leading to further improved properties. The designed structure was verified by determination of the crystal structures of consensus ARM proteins. Furthermore, variable positions have been determined to create a library, and a binder against a particular peptide has been selected (Varadamsetty *et al.*, manuscript in preparation).

Tetratricopeptide repeat proteins

An interesting characteristic of TPR modules, in contrast to the HEAT repeats, is that they do not undergo conformational changes upon ligand binding [30]. A consensus TPR motif was designed using amino acids with the highest statistical occurrence for each position, taking potentially co-evolving residues into consideration as well. Initially, three consensus proteins were constructed, comprising one, two or three repeats (CTPR1, CTPR2, and CTPR3) [31]. As confirmed in the 3D structure, these proteins adopt the typical TPR fold. Chemical denaturation and NMR-detected H/D-exchange studies revealed a stable core of an individual TPR motif. More repeats resulted in an increase in overall stability, an effect primarily due to a decrease in the rate of unfolding [32]. Similar results have been observed for the DARPins and natural ARs as well [23,33] and for the ARM repeat proteins (Varadamsetty *et al.*, manuscript in preparation) and may thus constitute a general property of repeat proteins.

Folding of TPR proteins could be described by the 1D-Ising model, in which each repeat is treated as an independent folding unit which is influenced by the folding state of its neighbors. For CTPR2 and CTPR3, the equilibrium folding–unfolding transition was shown to be sequential [34,35]. Recently, the non-two state unfolding behavior was confirmed in differential scanning calorimetry (DSC) studies of a complete series of CTPR proteins with 2–20 identical repeats [36]. The folding of full-consensus DARPins has also been evaluated by a 1D-Ising model, using evaluation by CD spectroscopy [23] or NMR at the single residue level [26•]. Their behavior can be described by a 1D-Ising model in a first approximation; nevertheless, their extreme stability limits the analysis of molecules with many repeats, as they cannot be unfolded at all. Therefore, H/D-exchange becomes dominated by local fluctuations.

Selection strategies for repeat proteins

To obtain specific binders, selections against the target of interest need to be carried out. In principle, all known selection technologies can be applied to these proteins. Regan and co-workers applied a split-GFP reassembly assay to screen libraries of TPR proteins binding to the target of interest [37•,38•,39]. The target peptide is genetically fused to one half of GFP, whereas the TPR library is genetically fused to the other half. TPR variants binding to the target can be identified by fluorescence in flow cytometry. So far, specific binders with μM affinities were obtained against different targets and characterized [37•]. However, this method might be limiting when high-affinity binders are the goal of selection. In general, *in vivo* selections do not seem to particularly favor high-affinity interactions: well expressed intracellular proteins are already at high concentrations (e.g. 120,000 molecules correspond to a concentration of 1 μM for a lymphocyte with a cell volume of 200 fL [40]), such that even low-affinity pairs will form complexes almost quantitatively.

The great majority of selections with DARPins have been carried out by using ribosome display, which is a technology fully taking place *in vitro*, leading to working library sizes of about 10^{12} [41,42], and which can be interfaced with error-prone PCR and off-rate selection to increase affinity and specificity [43]. Since DARPins fold well also *in vitro*, they may be especially suitable for this selection technology, typically requiring fewer rounds than scFv fragments [44,45]. Using ribosome display, DARPins were selected against a plethora of targets, such as kinases [46–48], membrane proteins [49–51] and IgE or its receptor FcεRIα [52–54]. For some DARPins, the affinity was further improved by applying error-protein PCR together with off-rate selections [51,55,56•].

DARPins are fast folding proteins [23]. As such, it was impossible to achieve a high level of display on the p3 phage coat protein with a variety of Sec-dependent signal sequences, since the DARPin-p3 fusion will fold before it is secreted to a form expressed in the inner *E. coli* membrane during filamentous phage assembly. Steiner *et al.* exchanged the Sec signal sequence for a signal recognition particle (SRP)-dependent one, which resulted in very efficient display of the DARPin molecule [57], just as efficient as slow folding proteins (e.g. scFv) with a Sec-dependent signal sequence. Using SRP phage display, specific DARPins with subnanomolar affinities against a number of targets, including EGFR and HER2, were selected [58]. This has opened the door to use phage display on whole cells to DARPins. Incidentally, previous attempts to achieve functional display via the Tat route have proven unsuccessful [59–61], as the full-length p3 protein may be incompatible with the Tat system. However, a truncated version of p3 can support Tat-mediated phage display [62]. Both SRP phage display and this finding may thus extend the application of phage display to proteins which fold fast in the cytosol.

New scaffolds, novel applications

The driving force behind the engineering of new binding proteins is formed by enabling new applications. New scaffolds are finding places in therapeutics, diagnostics, and numerous research applications, which benefit from the wide range of molecular formats, fusion proteins, and chemical conjugates which become possible with these robust proteins. The great majority of new applications have been developed with DARPins. We will concentrate on biomedical applications, but mention in passing that DARPins have also been used as crystallization chaperones (summarized in [63–65]).

Viral retargeting to tumors

Viral delivery systems for gene delivery will need to overcome the problem of cell-specific targeting and tissue-specific targeting. In previous experiments, using many different viruses, scFv fusions to viral surface proteins have been investigated; however, frequent incompatibilities with the viral assembly pathways were observed, leading to inefficient display of such targeting moieties.

DARPins have been successfully used in two viral retargeting applications now. Adenoviruses have been investigated as potential gene vectors for diagnostic or therapeutic applications for a long time. In this case, the virus is *not* expressing a DARPin on its surface, but a bispecific adapter was created which can be produced in *E. coli*. This might constitute a technology that can realistically be scaled up. In its most efficient form, it consists of four DARPins in tandem. Three of these DARPins bind and wrap around the trimeric knob domain at the end of the protruding adenovirus fibers; the fourth DARPin binds to the cellular target of interest, in this case HER2. The knob domain used was a mutant with reduced affinity to CAR, the natural receptor of adenovirus serotype 5 (Ad5), and adapter bound to the knob also prevented binding to CAR. The adapters showed a significant increase in transduction, measured by luciferase activity. This new strategy of altering the natural tropism of Ad5 with rationally designed adapters holds great promise for future developments in gene therapy [56•].

In another example, lentiviral vectors were retargeted to tumors by using DARPins [66], with the aim of destroying the tumor cells. For this purpose, the lentiviral vectors carried both the hemagglutinin (H) and the fusion protein (F) from measles virus, where the former was fused to different DARPins. All H-DARPin fusion proteins were efficiently expressed on the cell surface and incorporated into lentiviral vectors at a more uniform rate than scFvs, perhaps because of the more robust folding within the fusion protein. Indeed, the vectors only transduced HER2-positive cells. When applied systemically *in vivo*, these HER2-targeted lentiviral vectors showed exclusive gene expression in HER2-positive tumor tissue, whereas control vectors mainly transduced cells in spleen and liver.

Tumor diagnostics

DARPins can be employed in immunohistochemical diagnostics. Recently, G3, a HER2-specific DARPin with picomolar affinity, was tested in paraffin-embedded tissue sections and compared to an FDA-approved monoclonal antibody in tissue microarrays [67]. The data were correlated with HER2 amplification status measured by fluorescence *in situ* hybridization. It was found that the DARPin was able to detect a positive HER2 amplification status with similar sensitivity yet with higher specificity. Thus, DARPins form a valuable extension to the diagnostic toolbox; their potential in this field should be further explored.

Approaches to tumor therapy

In tumor therapy, the repeat protein scaffold can be used as the targeting moiety for a payload, or alternatively, the facile engineering of the protein might be exploited to create a multivalent DARPin with biological activity by itself.

A payload with a lot of potential is small interfering RNA (siRNA). DARPin C9, selected against the epithelial cell adhesion molecule (EpCAM), was used as a carrier for siRNA complementary to the *bcl*-2 mRNA, a pro-apoptotic factor [68]. C9 was genetically fused to protamine for complexation with the siRNA, and about 4–5 molecules could be bound per protamine. To increase uptake of siRNA, bivalent binders were constructed, either by using a flexible linker or by using a leucine zipper. For all tested constructs, a decrease in *bcl-2* expression was observed; this resulted in a significant sensitization of EpCAM-positive MCF-7 cells toward doxorubicin. The fusion proteins did not sensitize EpCAM-negative HEK293T cells, indicating that siRNA transfection at high specificity is achievable. The bivalent leucine zipper construct was more effective in the downregulation of *bcl*-2 than the dimer linked by a Gly-Ser linker, suggesting that it better matches the geometry of the receptor on the cell [68].

The EpCAM-specific DARPin Ec4 was fused to a truncated form of *Pseudomonas* exotoxin A (ETA″). The IC_{50} values on EpCAM-positive cells ranged from 0.005 pM to 0.7 pM on the positive tumor cells, whereas the IC_{50} value on negative control cells was 100,000-fold higher. *In vivo* fluorescence imaging demonstrated that Ec4-ETA″ labeled with Cy5.5 efficiently localized to tumors after systemic administration. Potent antitumor effects at well-tolerated doses were seen in mouse xenograft studies; some mice even showed complete regression of the tumor. However, tumor accumulation might be limited because of the short half-life of 11.2 min of the DARPin fusion protein in its current version [69].

Boersma *et al.* (manuscript in preparation) examined the biological activity of four previously selected EGFR-DARPins on A431 cells. By constructing a tetravalent, bispecific

DARPin, a molecule with activity in cellular assays at least similar to and in certain cases even greater than the FDA-approved antibody cetuximab was created. Thus, DARPins alone can have a biological effect; this increases the potential of DARPins as tumor targeting molecules.

Pharmacokinetics

Zahnd *et al.* recently investigated the effects of affinity and molecular size on the efficiency of tumor targeting and accumulation in a systematic study with HER2-specific DARPins in mouse xenografts [70••]. This work showed that there are *two* parameter regions for efficient tumor accumulation. The first, and perhaps unexpected, region is that of unmodified small DARPins, as long as they have a very high affinity. There was a direct and strong correlation between the total amount located at the tumor and the affinity of the DARPin; no leveling off with affinity was seen. For bivalent DARPins (avidity $\approx$ 10 pM), a *lower* accumulation in the tumor was seen than for the monovalent counterpart ($K_D \approx 90$ pM); the lower accumulation was also seen when this DARPin was fused to a nonbinding DARPin. This highlights the importance of small molecular weight (MW).

The second region of high tumor accumulation was with PEGylated DARPins. This modification increases their serum half-life. In this case, the importance of affinity for the extent of tumor uptake was less pronounced. Molecules of an intermediate size range (e.g. scFv) do not target as well as either of the extremes.

A possible explanation of these findings can be given if a very pronounced dependence of extravasation on MW is assumed, and that its cut-off is at lower MW than that of renal filtration. In this case, a molecule of intermediate MW would be filtered through the kidney, but still not extravasate well. However, a molecule of small MW needs to bind to its receptor on the tumor very tightly or it will be washed out rapidly. This affinity requirement is not as strong for very large, PEGylated molecules, which reside in the serum much longer.

It should be noted that these results are fully consistent with theoretical considerations from modeling studies [71]. However, they at first appear to be at variance with a series of elegant studies on monovalent and multivalent scFv fragments, which have been summarized to suggest that very high affinity is disadvantageous for tumor targeting [72]. However, Adams and co-workers used iodine as a label which is removed upon internalization, and thus additional affinity or avidity leading to more internalization will lead to *less* remaining label in the tumor [73]. In contrast, Zahnd *et al.* used a residualizing Tc label, which will *not* be removed and will thus be counted, no matter whether the protein has become internalized or remains on the surface. Thus, when considering *all* protein molecules that end up at the tumor, there appears to be no decrease in uptake with very high affinity. However, when counting only those which have remained *on the surface*, there is a decrease with very high affinity, since a larger proportion becomes internalized and looses its label. Thus, it is important to make these distinctions when discussing the influence of affinity on tumor uptake.

Intracellular applications

At this stage, it is unclear whether repeat proteins can be delivered *in vivo* at a level and in a format fit for controlling and influencing intracellular reactions, except by engineered viral delivery, as highlighted above. Nevertheless, exciting possibilities arise by the fact that repeat proteins can fold and remain active in the cytoplasm; they are currently mostly research tools though, requiring transfection of the DNA encoding the repeat protein.

TPR modules were designed to inhibit Hsp90. This chaperone is essential for the folding of many oncogenic proteins, for example, nuclear receptors as well as HER2; small molecule inhibitors are thus considered potential anticancer agents. Hsp90 itself only becomes functional when bound to Hsp-organizing protein (HOP), an interaction mediated by the TPR2A domain on HOP. The Hsp90-binding residues of TPR2A were grafted onto the previously discussed CTPR3 scaffold to introduce novel activities. The resulting molecule CTPR390 bound to Hsp90 with an affinity of 200 μM, whereas TPR2A's affinity for Hsp90 is 5 μM. From the 3D structure of CTPR390 in complex with the C-terminal peptide of Hsp90, it became clear that not all interactions seen in the TPR2A–Hsp90 complex were present in the CTPR390–Hsp90 complex [74]. By engineering charges on the back face the affinity increased to 1 μM. Treatment of BT474 cells with CTPR390 resulted in a decrease in HER2 levels with consequent inhibition of cell proliferation. This design provides new tools to further the knowledge of Hsp90-mediated protein folding [75].

Conclusions

Repeat protein applications, notably of DARPins, have seen a great rise over the last few years. They expand the application range beyond what is possible with antibodies, because of their robust biophysical properties and ease of production. The examples highlighted in this review have shown that there are many potential applications for these alternative scaffolds, whether as tumor targeting molecules, diagnostic tools, or in other therapeutic strategies. DARPins are currently in two Phase I/II clinical trials for ocular indications[a,b] and it will be exciting to see their further progress.

[a] http://clinicaltrials.gov/ct2/show/NCT01042678.

[b] http://clinicaltrials.gov/ct2/show/NCT01086761.

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Extensive H/D-exchange studies were performed on full-consensus DARPins, and they showed that the stability is greatly dependent on the coupling between repeats. The extraordinary slow exchange of protons (dominated by local fluctuations) indicates a very stable core structure in the DARPins. These findings will help in the design of highly stable DARPins for biomedical applications.

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